All-*Trans*-Retinoic Acid Induces Interleukin-8 via the Nuclear Factor-κB and p38 Mitogen-Activated Protein Kinase Pathways in Normal Human Keratinocytes

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Retinoic acid derivatives have been used successfully for the treatment of various dermatoses, such as psoriasis; however, topical application of these compounds often elicits skin irritation. We hypothesized that this irritation was as a result of the local production of interleukin-8 (IL-8). To test this hypothesis, we investigated whether all-*trans*-retinoic acid (ATRA) induced IL-8 production in normal human keratinocytes. Stimulation with 10^{-7} M ATRA enhanced IL-8 mRNA expression and induced IL-8 production. We also studied the intracellular signaling mechanisms of ATRA-induced IL-8 production in keratinocytes. ATRA increased the expression of RelA (p65), RelB, nuclear factor (NF)-κB2 (p52), and NF-κB1 (p50), and elevated the DNA-binding activity of p65 and phosphorylation of inhibitor κB (IκB) α. Introduction of a dominant-negative mutant of IκBα completely abolished ATRA-induced IL-8 production, which indicates that this process is NF-κB-dependent. We also studied the role of the p38 mitogen-activated protein kinase (MAPK) pathway in this phenomenon. ATRA phosphorylated the p38 MAPK, and SB202180 inhibited ATRA-induced IL-8 production, which indicates that the p38 MAPK is also involved in ATRA-induced IL-8 production. In summary, ATRA induces IL-8 production in both NF-κB- and p38 MAPK-dependent manners in normal human keratinocytes.

Key words: skin irritation/chemokine/p65/retinoid/IκBα/SB202180
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Retinoids include natural and synthetic compounds with specific biological activity similar to that of Vitamin A (retinol) (Orfanos *et al*, 1997). Retinol and its derivative acid are widely used to treat acne, psoriasis, and photo-aged skin. Despite their many beneficial effects, however, topical application of retinoids often causes local irritation that is manifested as redness, scaling, and dryness (Kang *et al*, 1995; Ale *et al*, 1997). The mechanism of retinoic acid-induced inflammation is poorly understood. The important role of interleukin-8 (IL-8) in the progress of irritation has been demonstrated (Schroder, 1995). A recent report showed that treatment with 2% retinol upregulated the level of IL-8 mRNA in the ear epidermis of BALB/c mice (Kim *et al*, 2003), and that anti-irritants significantly inhibited retinoid-induced IL-8 in cultured keratinocytes (Kim *et al*, 2003), indicating that IL-8 plays a central role in retinoid-induced skin irritation. Although human keratinocytes produce IL-8 in response to retinoids (Kim *et al*, 2003), the molecular mechanism involved is unclear.

IL-8 is a member of the C–X–C subfamily of chemokines (CXCL8; Zlotnik and Yoshie, 2000). As a key factor in the pathogenesis of inflammatory diseases, IL-8 has diverse biological properties, including chemotaxis of neutrophils and T lymphocytes (Baggiolini *et al*, 1989), regulation of cell adhesion (Djeu *et al*, 1990), activation of neutrophils (Mukaida *et al*, 1992), and modulation of histamine release (Kuna *et al*, 1991). Therefore, it seems worthwhile to investigate the potential role of IL-8 in retinoic acid-induced skin inflammation. Various cell types, such as endothelial cells, epithelial cells, synovial cells, T cells, fibroblasts, keratinocytes, chondrocytes, and some tumor cells, have been shown to produce IL-8 (Hoffmann *et al*, 2002). The classical inducers of IL-8 are inflammatory stimuli, such as IL-1, tumor necrosis factor (TNF)-α, bacterial lipopolysaccharides (LPS), 12-*O*-tetradecanoylphorbol-13-acetate, viruses, and double-stranded RNA (Hoffmann *et al*, 2002). IL-8 production is regulated at both the transcriptional and post-transcriptional levels. The 3′-end of the IL-8 transcript contains an AU-rich *cis*-element (ARE), which is responsible for the destabilization of a variety of cytokine mRNA (Shaw and Kamen, 1986). The sequence that spans nucleotides (nt) −1 to −133 within the 5′-flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene. The core IL-8 promoter contains a nuclear factor-κB (NF-κB) element that is required for activation in all of the cell types studied, and it also contains activating protein (AP)-1- and CCAAT/enhancer-binding protein (C/EBP)-binding sites. The latter two sites are dispensable for transcriptional activation in some cell types, but contribute to

Abbreviations: ATRA, all-*trans*-retinoic acid; CT, cycle threshold; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor κB; IL, interleukin; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NF-κB, nuclear factor κB; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RXR, retinoid X receptor; TNF, tumor necrosis factor

activation in other cell types. Thus, unlike the NF-κB site, the AP-1 and C/EBP sites are not essential for IL-8 gene induction, but are required for maximal gene expression (Hoffmann *et al*, 2002).

Cytokines and chemokines play important roles in the pathogenesis of various dermatoses. Based of the results of biochemical analyses and *in vitro* studies, it is clear that IL-8 contributes significantly to the pathological changes seen in psoriasis (Sticherling *et al*, 1991a, b, 1999; Takematsu and Tagami, 1993). The involvement of IL-8 in other dermatoses, such as bullous pemphigoid (Baroni *et al*, 2002), erythema migrans (Grygorczuk *et al*, 2002), atopic dermatitis, and allergic contact dermatitis (Albanesi *et al*, 2001), has also been demonstrated. The erythematous reaction in retinoid-induced skin irritation is clinically similar to a mild irritant dermatitis (Fisher *et al*, 1991; Ale *et al*, 1997; Grygorczuk *et al*, 2002). In particular, activated keratinocytes are an important source of chemotactic factors that direct the recruitment of specific leukocyte populations and regulate the quality, magnitude, and duration of the inflammatory response. Normal cultured human keratinocytes have been shown to produce IL-8 after appropriate stimulation (Larsen *et al*, 1989; Boorsma *et al*, 1994; Stoof *et al*, 2001).

Retinoic acid modulates immunological and inflammatory responses, most probably by regulating cytokine production, although it also affects epidermal cell growth and differentiation (Zitnik *et al*, 1994; Sawatsri *et al*, 2000). As retinoic acid upregulates IL-8 expression in fibroblasts (Zhang *et al*, 1992), neuroblastoma cells (Yang *et al*, 1993), and a human ovarian carcinoma cell line (Harant *et al*, 1995), we hypothesized that retinoic acid might also induce IL-8 production in human keratinocytes. To test this hypothesis, we investigated whether all-*trans*-retinoic acid (ATRA) was able to induce IL-8 production in cultured normal human keratinocytes, and we assessed the involvement of the NF-κB and p38 mitogen-activated protein kinase (MAPK) pathways in this process.

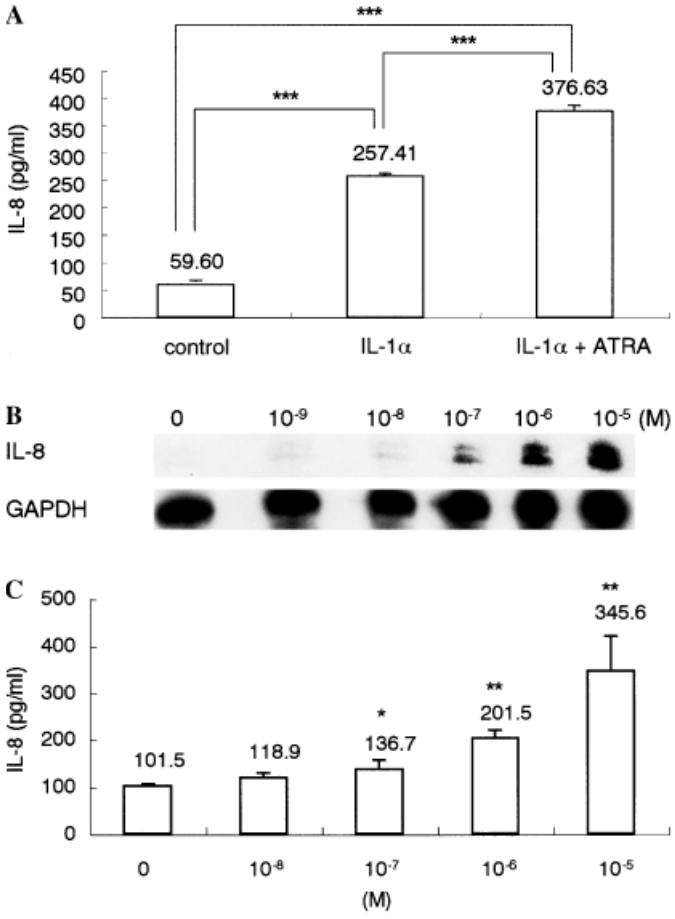

Figure 1
All-*trans*-retinoic acid (ATRA) simulates the production of interleukin (IL)-8 by human keratinocytes. (*A*) Subconfluent keratinocyte cultures were treated with IL-1α (1 ng per mL), ATRA (10^{-6} M), or vehicle (dimethyl sulfoxide). After 36 h of incubation, the culture supernatants were collected and subjected to ELISA for the detection of IL-8. (*B*) Keratinocytes were stimulated with ATRA (10^{-9}–10^{-5} M). IL-8 mRNA expression was monitored by RT-PCR after a 24-h incubation. (*C*) Keratinocytes were stimulated with ATRA for 36 h, and the level of secreted IL-8 was measured by ELISA.

Results

ATRA increases the production of IL-8 in keratinocytes IL-1α is the prominent inducer of IL-8 (Kristensen *et al*, 1991; Hoffmann *et al*, 2002). Moreover, retinoids reduce IL-1 induction of IL-8 production in human monocytes (Gross *et al*, 1993). Therefore, we initially investigated the effect of ATRA on IL-1α-induced IL-8 production. Keratinocytes were treated with IL-1α (1 ng per mL) or IL-1α plus ATRA (10^{-6} M), and the IL-8 levels in the culture supernatants were measured after 36 h of incubation. Marked induction of IL-8 was observed in IL-1α-stimulated cells, and ATRA increased IL-8 production in a synergistic manner (Fig 1*A*).

To assess the effect of ATRA on IL-8 production in keratinocytes, we stimulated the keratinocytes with different concentrations of ATRA. IL-8 mRNA expression was scarcely detectable by the ribonuclease protection assay (RPA) in samples that contained the vehicle (dimethyl sulfoxide (DMSO)) alone (Fig 1*B*). After 24 h of treatment with various doses of ATRA (10^{-9}–10^{-5} M), IL-8 mRNA expression was increased in a concentration-dependent manner (Fig 1*B*). The release of IL-8 into the culture medium was upregulated in a concentration-dependent manner, and was consistent with the increase in IL-8 mRNA (Fig 1*C*).

ATRA increases the expression of the NF-κB family members in human keratinocytes NF-κB binding is essential for the activation of IL-8 gene transcription (Kunsch *et al*, 1994). Several members of the NF-κB family, such as p50 (NF-κB1), p65 (RelA), c-Rel, and p52 (NF-κB2), have been shown to bind to the NF-κB motif of the IL-8 promoter (Kunsch and Rosen, 1993; Stein *et al*, 1993; Harant *et al*, 1996a). To characterize the molecular mechanisms underlying ATRA-induced IL-8 production in keratinocytes, the expression of NF-κB family members in ATRA-treated keratinocytes was examined using the RPA. The expression levels of p65, RelB, p52, and p50 mRNA started to increase 1 h after the addition of ATRA, and persisted for more than 24 h (Fig 2). Although c-Rel was detected faintly 12–48 h after ATRA treatment, the basal level of expression of c-Rel mRNA was very low in the RPA.

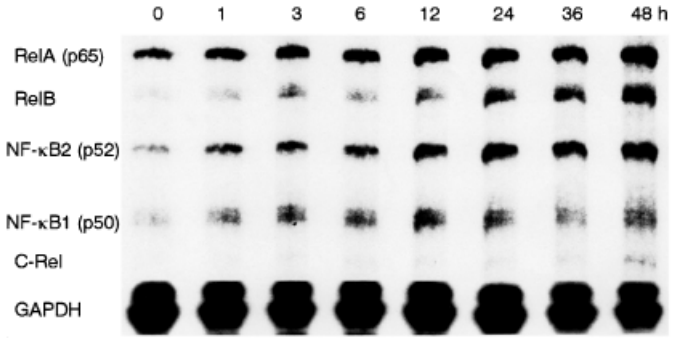

Figure 2
All-*trans*-retinoic acid (ATRA) increases the expression of nuclear factor (NF)-κB subunit mRNA. Keratinocytes were exposed to 10^{-6} M ATRA, and total RNA samples were collected at the indicated time points. The expression levels of the NF-κB subunit mRNAs were examined using the ribonuclease protection assay.

ATRA increases the DNA-binding activity of p65 in human keratinocytes We also examined the DNA-binding activities of the NF-κB family members in the nuclear extracts of ATRA-stimulated keratinocytes. The DNA-binding activity of p65 increased between 6 and 24 h post-treatment with ATRA (Fig 3*A*). In addition, ATRA upregulated the DNA-binding activity of p65 in a concentration-dependent manner (Fig 3*B*). In contrast, the DNA-binding activity of p50 was not influenced by ATRA. The DNA-binding activity of the c-Rel protein was not detected in either ATRA-treated or untreated keratinocytes (data not shown).

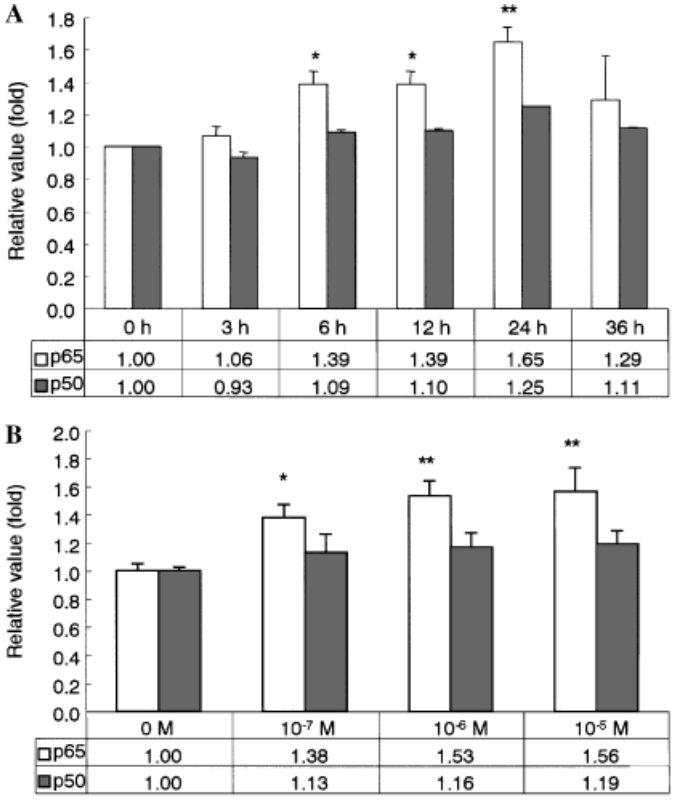

Figure 3
Nuclear translocation of p65 is increased by all-*trans*-retinoic acid (ATRA) treatment. (*A*) Keratinocytes were stimulated with ATRA (10^{-6} M), and the nuclear proteins were extracted at the indicated time points. An enzyme-linked immunoassay was performed to monitor the DNA-binding activities of the nuclear factor (NF)-κB subunits in the nucleus. The relative values for the optical density at 655 nm are normalized to the value of 0 h as 1 U, and are plotted on the graph. (*B*) ATRA (10^{-9}–10^{-5} M) was added to subconfluent-conditioned keratinocytes, and the cultures were incubated for 24 h. The DNA-binding activities of the NF-κB subunits in the nucleus were examined, and the relative values are normalized to the value for 0 M, which is designated as 1 U. $^{*}p<0.05$; $^{**}p<0.01$.

ATRA phosphorylates IκBα in cultured human keratinocytes The inhibitory molecule IκB anchors NF-κB in the inactive form in the cytosol (Baeuerle and Henkel, 1994). A crucial step in NF-κB signal transduction involves the dissociation of NF-κB from IκB, which is followed by NF-κB translocation to the nucleus (Baeuerle and Henkel, 1994).

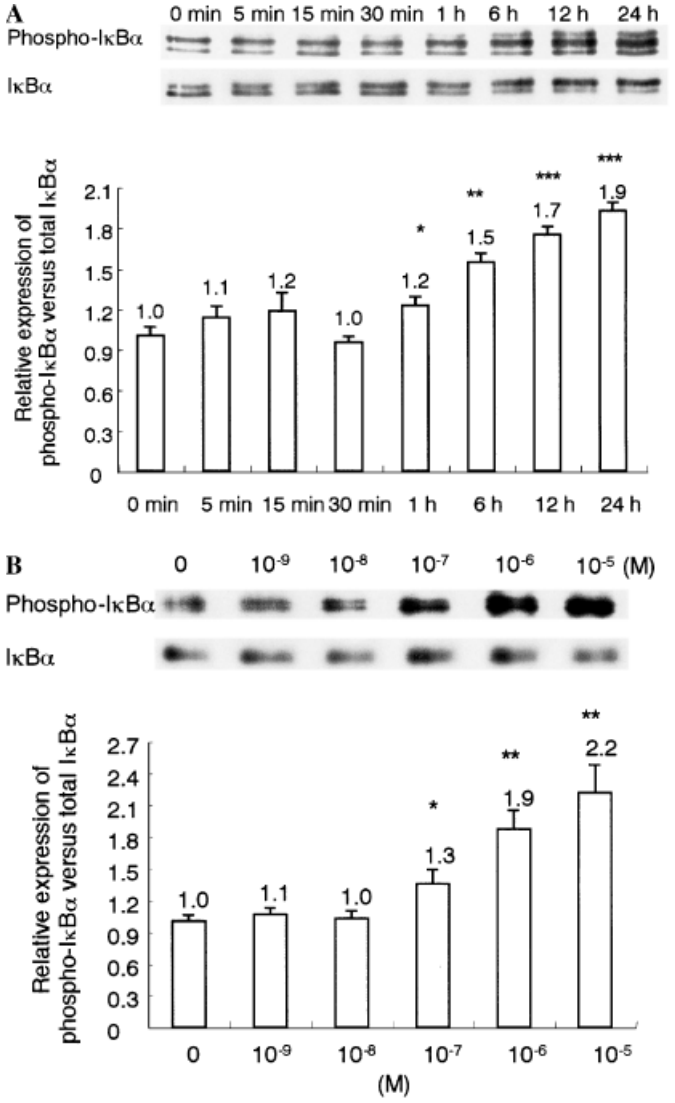

Figure 4
All-*trans*-retinoic acid (ATRA) increases the level of phosphorylated IκBα in human keratinocytes. (*A*) Keratinocytes were stimulated with ATRA (10^{-6} M), and the cellular proteins were extracted at the indicated time points. Immunoblotting was performed with anti-IκBα and anti-phospho-specific IκBα antibodies. The relative levels of phosphorylated IκBα are estimated using the total level of IκBα protein as the reference, and these values are normalized against the value at 0 min, which is designated as 1 U. (*B*) ATRA (10^{-9}–10^{-5} M) was added and incubated for 24 h. The relative levels of phosphorylated IκBα protein expression are estimated from the immunoblots. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

The dissociation of NF-κB from IκB requires the phosphorylation of IκB, which results in rapid and ubiquitous degradation of IκB. Thus, the effect of ATRA on the expression and phosphorylation of IκBα was investigated. ATRA increased the level of phosphorylation of IκBα, although ATRA had no significant effect on the total level of IκBα protein (Fig 4*A* and *B*). The relative level of expression of phosphorylated IκBα to total IκBα was increased significantly, 6 h post-ATRA treatment, and had increased 1.9-fold at 24 h (Fig 4*A*). In our concentration-dependency study, 24 h of ATRA treatment increased the level of phosphorylated IκBα 1.3-fold at 10^{-7} M, 1.9-fold at 10^{-6} and 2.2-fold at 10^{-5} M, as compared with vehicle-treated keratinocytes (Fig 4*B*).

Mutant IκBα abrogates the production of IL-8 by ATRA in human keratinocytes To determine whether ATRA-induced IL-8 production in keratinocytes was dependent on NF-κB, we introduced mutated-IκBα (IκBαM) gene using an adenovirus vector (AxIκBαM), which results in the blockade of NF-κB signals by unphosphorylated IκBα (Brown *et al*, 1995; Chen *et al*, 1995). Keratinocytes were transfected with AxIκBαM or AxLacZ, and stimulated with ATRA. The culture supernatants were collected at 12, 24, and 48 h after the addition of ATRA. The ELISA demonstrated that IκBαM not only abrogated ATRA-dependent production of IL-8, but also drastically suppressed the basal production of IL-8 (Fig 5*A*). IκBαM also suppressed IL-8 mRNA expression in both ATRA-treated and untreated keratinocytes (Fig 5*B*). The increased expression of IκBα protein in IκBαM-transfected keratinocytes confirms that mutated IκBα persists in the cell and suppresses the NF-κB signal (Fig 5*C*).

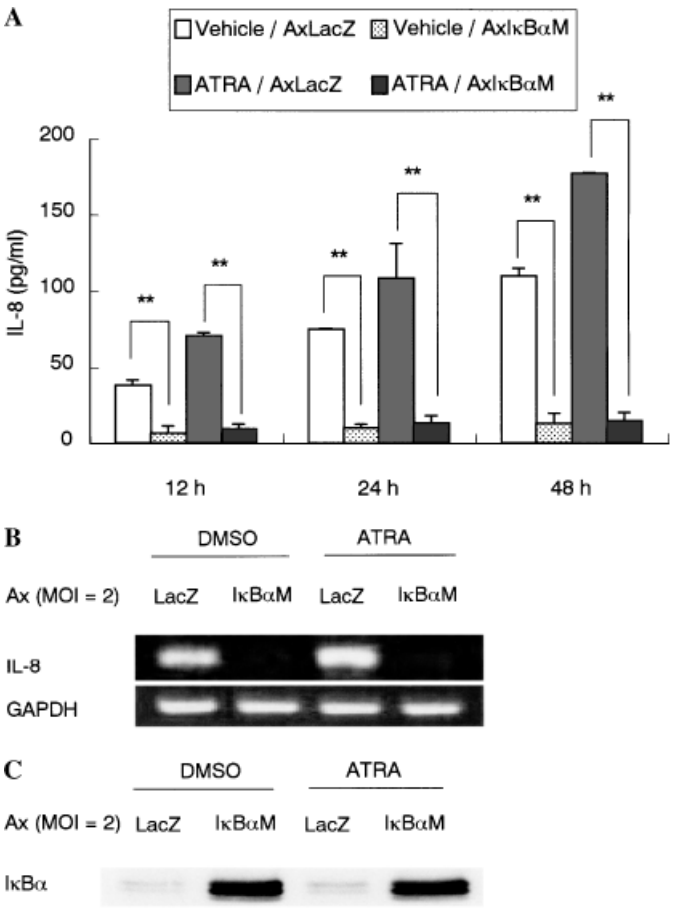

Figure 5
Blockade of nuclear factor (NF)-κB signaling abrogates the induction of interleukin (IL)-8 by all-*trans*-retinoic acid (ATRA). (*A*) Keratinocytes were transfected with AxIκBαM or AxLacZ at a multiplicity of infection (MOI) = 2 for 12 h before the addition of ATRA. ATRA (10^{-6} M) or vehicle was then added, and the cultures were incubated for the indicated time periods. The culture supernatants were collected, and the levels of IL-8 protein were measured. $^{*}p<0.05$; $^{**}p<0.01$. (*B*) Keratinocytes were transfected with adenovirus vector and stimulated with ATRA (10^{-6} M) or vehicle. The levels of IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression 24 h post-treatment with ATRA were analyzed by RT-PCR. (*C*) Keratinocytes were transfected with adenovirus vector and stimulated with ATRA (10^{-6} M) or vehicle. The levels of IκBα protein expression 24 h after ATRA treatment were analyzed by western blotting.

The p38 MAPK inhibitor SB202180 partially suppresses ATRA-induced IL-8 expression in human keratinocytes We investigated whether the p38 pathway was also involved in ATRA-induced IL-8 gene expression in keratinocytes. After the addition of ATRA, the phosphorylation of p38 increased, whereas the total level of p38 protein was unchanged (Fig 6*A*). The phosphorylation of p38 started at 5 min, peaked at 1 h, and persisted for 24 h post-treatment with ATRA (Fig 6*A*). ATRA-dependent p38 phosphorylation was inhibited by SB202180 (Fig 6*B*). The cells were treated with 5, 10, or 20 μM of SB202180 for 2 h before the addition of ATRA, and the keratinocytes were then incubated for 36 h. ATRA-induced IL-8 production was inhibited in a concentration-dependent manner by SB202180 (Fig 6*C*). Consistent with the decrease in IL-8 production, the levels of ATRA-induced IL-8 mRNA expression were also decreased in a concentration-dependent manner by SB202180 (Fig 6*D*). In this study, SB202180 did not affect the basal level of IL-8 production (data not shown).

Discussion

In this study, we demonstrated that ATRA induces IL-8 production in cultured normal human keratinocytes. Skin irritation following topical application of retinoic acid at least partially explained by the local induction of IL-8 production by epidermal keratinocytes (Kim *et al*, 2003). IL-1α increases IL-8 production in normal human keratinocytes (Kristensen *et al*, 1991; Hoffmann *et al*, 2002), and ATRA increased IL-8 production with IL-1α synergistically (Fig 1*A*). This result is contrary to that of a previous report, in which IL-1-induced IL-8 production by human monocytes was markedly reduced by retinoids (Gross *et al*, 1993). Nevertheless, LPS-induced IL-8 production was increased by ATRA in a same cell system (Gross *et al*, 1993). In addition, the synergistic effects of ATRA and TNF-α and 12-*O*-tetradecanoylphorbol-13-acetate on IL-8 gene activation have been reported for the human melanoma cell line G-361 (Harant *et al*, 1996b) and the human acute promyelocytic leukemia cell line (Atkins and Troen, 1995), respectively. Therefore, the effects of ATRA on IL-8 production vary, according to both cell type and the co-administered reagent.

Retinoic acid exerts its pleiotropic effects through binding to two groups of nuclear receptors, the retinoic acid

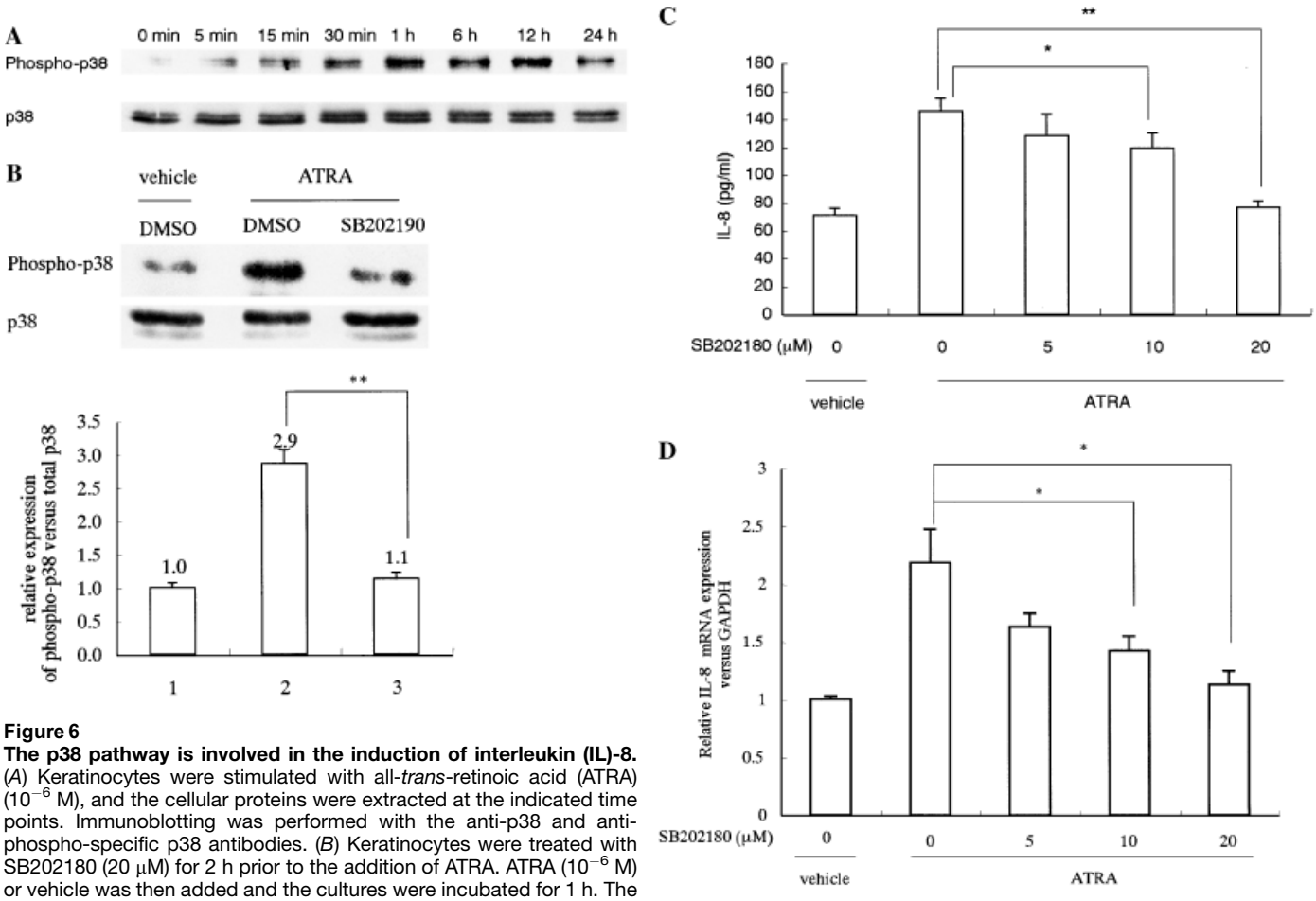

Figure 6
The p38 pathway is involved in the induction of interleukin (IL)-8. (*A*) Keratinocytes were stimulated with all-*trans*-retinoic acid (ATRA) (10^{-6} M), and the cellular proteins were extracted at the indicated time points. Immunoblotting was performed with the anti-p38 and anti-phospho-specific p38 antibodies. (*B*) Keratinocytes were treated with SB202180 (20 μM) for 2 h prior to the addition of ATRA. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 1 h. The expression levels of p38 and phospho-p38 were examined by western blotting. The intensity of each band was quantified as described in Materials and Methods**,** and the graph was made to show the significant effect of p38 inhibitor on ATRA-induced phospho-p38. (*C*) Keratinocytes were treated with various concentrations of SB202180 (0–20 μM) for 2 h. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 36 h. The levels of IL-8 in the culture supernatants were determined by ELISA. $^{*}p<0.05$; $^{**}p<0.01$. (*D*) Keratinocytes were treated with various concentrations of SB202180 (0–20 μM) for 2 h. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 18 h. The relative IL-8 mRNA expression levels were assessed by real-time quantitative RT-PCR.

receptors (RAR-α, -β, -γ) and the retinoid X receptors (RXR-α, -β, -γ) (Giguere *et al*, 1987; Petkovich *et al*, 1987; Benbrook *et al*, 1988; Brand *et al*, 1988; Krust *et al*, 1989). RAR usually form heterodimers with RXR (Chambon, 1996), and these dimers are able to bind to specific DNA sequences, which are known as retinoic acid-responsive elements (RARE). RAR–RXR complexes act as ligand-inducible transcription factors. The consensus sequence of RARE contains hexanucleotide half-sites that are arranged as inverted or direct repeats, spaced by various numbers of nt (de The *et al*, 1990); however, the IL-8 gene does not contain the classical ATRA response elements (Baggiolini and Clark-Lewis, 1992). Therefore, IL-8 induction by ATRA may not depend on this "classical pathway". In certain cases, retinoic acid has been shown to regulate genes that do not contain classical RARE motifs (Gudas *et al*, 1994). These genes can be regulated by retinoic acid via secondary events, such as the induction and activation of NF-κB (Segars *et al*, 1993). It has been shown that the induction of major histocompatibility class I genes by retinoic acid in human embryonal carcinoma NTera2 (NT-2) cells involves both the activation of RAR–RXR heterodimers and the induction of the NF-κB molecules p50 and p65 (Segars *et al*, 1993). The binding of NF-κB to the IL-8 promoter is essential for constitutive activation of IL-8 gene transcription (Hoffmann *et al*, 2002), and p65 is one of the major components responsible for κB binding to the IL-8 promoter (Kunsch and Rosen, 1993). ATRA is able to induce IL-8 gene expression by increasing NF-κB transcriptional activity in some cells (Harant *et al*, 1996a; Chang *et al*, 2000). In airway epithelium cells, ATRA-enhanced NF-κB binding to the promoter region involves p65 and p50 (Chang *et al*, 2000). In the human melanoma cell line G-361, the synergistic effect of ATRA and TNF-α on IL-8 expression is dependent on their combined activation of the IL-8 promoter, which requires an intact NF-κB-binding site (Harant *et al*, 1996b). In this study, we show nuclear translocation of p65 and IκB phosphorylation in ATRA-treated keratinocytes. Therefore, ATRA-dependent IL-8 induction probably involves increased levels of p65 (Segars *et al*, 1993). Similar effects of ATRA on NF-κB subunits have been observed in other cell types (Segars *et al*, 1993; Feng and Porter, 1999; Farina *et al*, 2002). ATRA-mediated increases in the levels of p65 and p50 transcripts are dependent on the "classical pathway".

An alternative mechanism for IL-8 induction via NF-κB signaling is the phosphorylation of IκBα. Unfortunately, the molecular mechanism by which ATRA phosphorylates IκBα is unclear. The introduction of IκBαM completely abolished

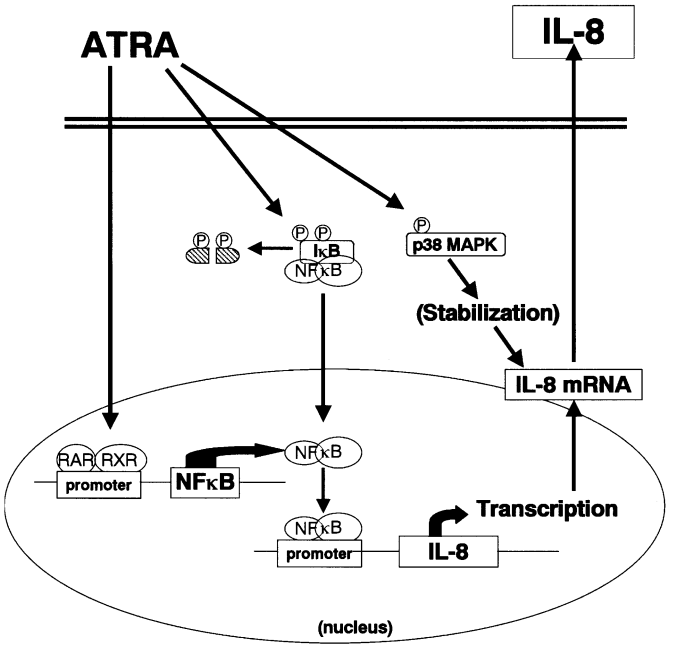

Figure 7
Schematic representation of a possible mechanism for all-*trans*-retinoic acid (ATRA)-dependent induction of interleukin (IL)-8 production by keratinocytes. A possible mechanism for ATRA-dependent induction of IL-8 production by keratinocytes is presented. ATRA phosphorylates IκBα, which causes nuclear translocation of nuclear factor (NF)-κB elements, and results in the transactivation of the NF-κB signal. The increased levels of p65 and p50 induced by ATRA may also contribute to activation of the NF-κB signal. Activation of the p38 mitogen-activated protein kinase (MAPK) pathway may play a role in IL-8 mRNA stabilization and IL-8 production.

IL-8 production and reduced the basal level of IL-8 production. These findings suggest that NF-κB activation is essential in ATRA-induced IL-8 production in normal keratinocytes.

Activation of p38 MAPK was elicited by ATRA in keratinocytes. The finding that the p38 inhibitor SB202180 specifically decreases ATRA-induced IL-8 production suggests that the p38 MAPK is also involved in ATRA-induced IL-8 production. A previous report has demonstrated that the p38 MAPK pathway contributes to cytokine/stress-induced IL-8 gene expression by stabilizing mRNA through an ARE-targeted mechanism at the post-transcriptional level (Hoffmann *et al*, 2002). Therefore, ATRA may stabilize IL-8 mRNA by activating the p38 pathway, thereby enhancing IL-8 production in keratinocytes. In summary, ATRA induces IL-8 production in normal human keratinocytes via NF-κB and the p38 MAPK signal pathways.

Materials and Methods

Cell culture Normal human keratinocytes were cultured in MCDB153 medium that was supplemented with insulin (5 μg per mL), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg per mL), and Ca^{2+} (0.03 mM), as described previously (Yamasaki *et al*, 2003a). The study was conducted according to Declaration of Helsinki principles. All of the procedures that involved human subjects received prior approval from the Ethical Committee of Ehime University School of Medicine, and all the subjects provided written informed consent.

Reagents ATRA was purchased from Sigma Chemical Co (St Louis, Missouri), and was dissolved in DMSO. Recombinant human IL-1α was a generous gift from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The anti-IκBα and anti-phospho-IκBα antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, Massachusetts). The p38 MAPK inhibitor SB202180 was purchased from Calbiochem–Novabiochem International Co. (San Diego, California), dissolved in DMSO at a concentration of 2 mM, and stored at −20°C.

Adenovirus construction and infection The cosmid cassette pAxCAw (Miyake *et al*, 1996) and the parental virus Ad5-dlX (Miyake *et al*, 1996) were kind gifts from Dr Izumu Saito (Tokyo University, Japan). The full-length coding region of the IκBαM cDNA was obtained from the *Hind*III- and *Bam*HI-digested fragments of pCMV-IκBαM (BD Biosciences Clontech, Palo Alto, California), and subcloned into the cosmid cassette pAxCAw. IκBαM encodes the dominant negative mutant form of IκBα, which is a non-degradable form of human IκBα, in which serines 32 and 36 are replaced by alanine residues (S32A/S36A), thereby blocking its phosphorylation and degradation (Brown *et al*, 1995; Chen *et al*, 1995). The NF-κB signal cannot be activated in cells that over-express IκBαM, regardless of the presence of extracellular stimuli that normally induce the phosphorylation of IκBα and activation of the NF-κB signal (Brown *et al*, 1995; Feig *et al*, 1999). An adenovirus vector that contains the CAG promoter and IκBαM (AxIκBαM) was generated using the cosmid cassettes and Ad DNA-TPC (COS-TPC) method, and virus stocks were prepared using the standard procedure (Miyake *et al*, 1996). Concentrated, purified virus stocks were prepared by CsCl gradient centrifugation, and the virus titer was estimated in a plaque formation assay.

Subconfluent cultures of normal human keratinocytes were infected with AxIκBαM at a multiplicity of infection (MOI) of 2, and AxLacZ was used as the control vector. The vectors were cultured with the cells for 60 min, with a brief period of agitation every 15 min. This was followed by the addition of fresh culture medium, and re-incubation for 12 h, before treatment with ATRA. The expression of these genes was confirmed by RT-PCR using specific probes.

Oligonucleotide probe preparation PCR-amplified human cDNAs were inserted into the *Eco*RI and *Hind*III sites of the pPMG vector (BD Pharmingen, San Diego, California). The inserted cDNA corresponded to the following sequences: nt 412–746 of RelA (p65) (GenBank/EBI accession no. NM021975); nt 709–1013 of RelB (NM006509); nt 804–1074 of NF-κB2 (p52/p100) (NM002502); nt 877–1122 of NF-κB1 (p50/p105) (NM003998); nt 1758–1982 of c-Rel (X75042). The pPMG vector (Pharmingen), which incorporates the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, was used as the internal standard.

RPA Total RNA was isolated from cultured human keratinocytes using Isogen (Nippon Gene, Toyama, Japan). Single-stranded antisense riboprobes were prepared by *in vitro* transcription of human cDNA fragments using the RiboQuant *In Vitro* Transcription Kit (Pharmingen) in the presence of [α-^{32}P] UTP. The hCK-5 probe (Pharmingen) for IL-8 detection and an oligonucleotide probe for NF-κB were used as the templates for the *in vitro* transcription reaction. The hybridization products were separated on a gel, and exposed to photographic film, as described previously (Yamasaki *et al*, 2003b). At least three independent studies were performed, with similar results. One representative experiment is shown in each figure. The GAPDH bands appear as a doublet, as reported previously (Dai *et al*, 2004), which may be because of the fact that the end of the GAPDH mRNA is highly susceptible to RNase digestion, even though it is double stranded.

RT-PCR Total RNA samples from cultured cells were isolated at the indicated time points using Isogen (Nippon Gene Co.). RT-PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The cDNA was gen-

erated from reverse transcription of total RNA for 30 min at 60°C, and then heated to 94°C for 2 min. The PCR cycle consisted of 1 min at 94°C for denaturation, and 1.5 min at 60°C for annealing and primer extension. The IL-8 sequences were amplified for 34 cycles, and the GAPDH sequences for 22 cycles. The following primer pairs were used: human IL-8, 5′-CTTCTCTGCAGCACATCC-3′ and 5′-AAGACCTCTCAAGGCTTTG-3′; and GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR products were visualized on 2% agarose gels that contained ethidium bromide. The PCR products were sequenced, to confirm the accuracy of amplification.

Real-time quantitative PCR Total RNA samples from cultured cells were isolated using Isogen (Nippon Gene Co.). Real-time RT-PCR was performed in the ABI PRISM 7700 sequence detector (PE Applied Biosystems, Branchburg, New Jersey). The Pre-Developed TaqMan assay reagents (set of primers and probe) for human IL-8 (Accession Numbers: NM_000584) and for human GAPDH endogenous control (Accession Numbers: NM_002046) were purchased from Applied Biosystems (Foster City, California). The RNA analysis was undertaken using the TaqMan One-Step RT-PCR Master Mix reagents kit (PE Applied Biosystems) and 500 ng of total RNA. Thermal cycling was initiated at 48°C for 30 min for reverse transcription reaction, followed by a first denaturation step at 95°C for 10 min, and then 40 cycles PCR at 95°C for 15 s and at 60°C for 1 min according to the manufacturer's instructions using 2 × AmpliTaq Gold DNA Polymerase mix (25 μL), 40 × RT enzyme mix (1.25 μL), 20 × Pre-Developed TaqMan assay reagent (2.5 μL). All one-step RT-PCR reactions were performed in 50 μL reaction volumes, and carried out in a 96-well plate. This experiment used two dye layers to detect the presence of target and control sequences. The 6-FAM dye layer yielded the results for quantification of IL-8 mRNA, and the VIC dye layer yielded the results for quantification of GAPDH. Quantification of gene expression was performed using the comparative cycle threshold (CT) method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to GAPDH gene. To calculate the fold change (increase or decrease), the CT of the housekeeping gene (GAPDH) was subtracted from the CT of the target gene (IL-8) to yield the ΔCT. Change in expression of the normalized target gene as a result of an experimental manipulation was expressed as $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT$ sample $-$ ΔCT control as described previously (Babu and Nutman, 2003). In this study, each assay was performed in triplicate, and the fold change of each sample was normalized against that of the vehicle as 1 U.

ELISA Culture supernatants were collected at the indicated time points after treatment, and stored at −70°C until used for ELISA. The IL-8 ELISA kit was purchased from R&D Systems (Minneapolis, Minnesota), and used according to the manufacturer's instructions. The optical density at 450 nm was measured with an Immuno Mini NJ-2300 microplate reader (Nalge Nunc International K.K., Tokyo, Japan). All of the assays were performed at least three times.

Western blot analysis The cells were harvested by scraping into extraction buffer that contained 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The analysis was performed using the Vistra ECF Kit (Amersham Biosciences K.K., Tokyo, Japan) and the FluorоImager (Molecular Dynamics Inc., Sunnyvale, California), as described previously (Yamasaki *et al*, 2003b). At least three independent studies were performed, with similar results. One representative experiment is shown in each of the figures. The intensity of each band was quantified using ImageQuant (Molecular Dynamics Inc.), with reference to the control signal, which was assigned the value of 1 U. Reproducibility was confirmed by performing three independent experiments.

Nuclear protein preparation and detection of transcription factor activity Nuclear proteins were prepared according to the instructions provided in the TransFactor Extraction Kit (BD Biosciences Clontech, Palo Alto, California). Briefly, cells were harvested in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) that contained protease inhibitors. Nuclear pellets were collected by centrifugation, and resuspended in extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT) that contained protease inhibitors. Finally, the protein concentrations of the nuclear extracts were determined, and the samples were stored at −70°C until used.

DNA-binding activities of the NF-κB family members were determined using the BD Mercury Transfactor Kits (BD Biosciences Clontech), according to the manufacturer's instructions. The relative optical density values at 655 nm were normalized to the control values (at 0 h or for 0 M), which were designated as 1 U, and plotted on a graph. All of the assays were performed on at least three separate occasions.

Statistical analyses The data were collected from at least three independent experiments. Quantitative data are expressed as the mean ± SE. Statistical significance was determined by the paired Student's *t* test. Differences were considered to be statistically significant for $p < 0.05$. The levels of statistical significance are indicated, as follows, in the figures: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

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